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Fine Tuning of the Threshold of T Cell Selection by the Nck Adapters

Edwige Roy,*† Dieudonné Togbé,*† Amy Holdorf,§‡§ Dmitry Trubetskoy,*† Sabrina Nabti,*† Günter Küblbeck,‡ Sabine Schmitt,‡ Annette Kopp-Schneider,‡ Frank Leithäuser,* Peter Möller,* Friedhelm Bladt,‡§ Günter J. Hämmerling,‡ Bernd Arnold,* Tony Pawson,‡ and Anna Tafuri*†‡

Thymic selection shapes the T cell repertoire to ensure maximal antigenic coverage against pathogens while preventing autoimmunity. Recognition of self-peptides in the context of peptide-MHC complexes by the TCR is central to this process, which remains partially understood at the molecular level. In this study we provide genetic evidence that the Nck adapter proteins are essential for thymic selection. In vivo Nck deletion resulted in a reduction of the thymic cellularity, defective positive selection of low-avidity T cells, and impaired deletion of thymocytes engaged by low-potency stimuli. Nck-deficient thymocytes were characterized by reduced ERK activation, particularly pronounced in mature single positive thymocytes. Taken together, our findings identify a crucial role for the Nck adapters in enhancing TCR signal strength, thereby fine-tuning the threshold of thymocyte selection and shaping the preimmune T cell repertoire. The Journal of Immunology, 2010, 185: 000–000.

Thymic ontogeny leads to the generation of functionally competent T cells through somatic rearrangement of the TCRα and TCRβ loci and selective maturation of the thymocytes, whose TCR interacts with self peptide-MHC (pMHC) complexes within a defined range of avidity. Thymic development proceeds through discrete stages, which can be differentiated on the basis of the configuration of the TCRα and TCRβ loci and of the expression of the CD4 and CD8 coreceptors. First, the productive rearrangement of the TCRβ locus in CD4−CD8− (double negative; DN) thymocytes leads to expression of the rearranged TCRβ-chain with the invariant pre–Tα-chain. The concerted actions of the CD3 signalosome and the Src family and Syk/ZAP-70 tyrosine kinases (1) are then required for progression to the CD4+CD8+ (double positive; DP) stage and expansion through a series of rapid cell divisions. During the DP stage, rearrangement of the TCRα-chain results in the expression of the mature TCRαβ complex. The TCR ability to discriminate between small amino acid variations in antigenic peptides controls repertoire selection and induction of self-tolerance (2). Only TCR–pMHC interactions within a relatively narrow range of functional avidity lead to thymocyte differentiation into mature T cells. In contrast, weak TCR–pMHC binding causes “death by neglect,” while high-avidity interactions result in clonal deletion, anergy, clonal diversion, or receptor editing, a series of events collectively responsible for the induction of central tolerance.

The role of the Nck adapters in T cell development has been inferred from their interaction with key players in TCR signaling, such as SLP-76 and CD3ε, but has not been genetically demonstrated. The Nck adapter family (3) comprises two members, Nck1 and Nck2, which are highly homologous and structurally characterized by one Src homology (SH)2 domain and three SH3 domains. Their wide tissue distribution includes developing and mature T cells. In a variety of cell types, the Nck adapters act as a molecular link between phosphotyrosine signals and regulation of the actin cytoskeleton (4–6), as the Nck SH2 domain is recruited to receptor tyrosine kinases or tyrosine phosphorylated docking proteins, and the SH3 domains bind to proteins controlling actin cytoskeletal organization (3), such as the Wiskott-Aldrich syndrome protein (WASP), WA VE-1, and the p21 activated kinase.

In T cells, biochemical (7) and live colocalization (8) analyses in transfected Jurkat T cells support the hypothesis that Nck recruitment to the immune synapse is phosphorylation dependent and is mediated by binding of its SH2 domain to phospho–SLP-76. Upon formation of a trimolecular complex, Nck–SLP-76–Vav1, Nck would recruit WASP, thereby promoting actin cytoskeletal rearrangement (9). Pull-down experiments in resting and activated Jurkat T cells suggest that the first SH3 domain of Nck binds to the proline rich sequence (PRS) of CD3ε upon TCR engagement. In this context, a “closed” conformation of CD3ε, masking the PRS and preventing its interaction with Nck (10), would account for the inability of Nck to bind to CD3ε from resting T cells. Biochemical and structural studies have shown that a noncanonical PxxDIY ε170 region juxtaposed to the PRS may also be crucial for Nck–CD3ε interaction and functionally relevant as Nck binding prevented Fyn-mediated Yε170 phosphorylation, and Yε170 phosphorylation resulted in Nck displacement (11). Notably,
the characterization of mice deprived of the CD3ε.PRS region has shown no effects on the development and function of the polyclonal T cell repertoire (12), whereas alterations in the development (13) and responsiveness (14) of CD3ε.PRS mutant T cells have been reported in CD3ε.PRS mutant mice expressing different transgenic TCRs. The Nck adapters are essential to enhance mature T cell sensitivity and reactivity to TCR-mediated stimulation (15). However, their role in T cell development is unknown. To address this issue, we used a conditional genetic system (5, 16, 17) in combination with two TCR transgenic models of different avidity for endogenous ligands. Our findings indicate that the Nck adapters are essential for positive and negative selection upon TCR engagement by low-avidity ligands but are not required for selection of high-avidity T cells. Nck-deficient thymocytes exhibited enhanced CD3ε phosphorylation and impaired ERK activation. Taken together, our findings provide genetic evidence that the Nck adapters regulate the threshold of thymic selection, thereby contributing to shape the preimmune T cell repertoire.

Materials and Methods

**Mice**

Nck1<sup>-/-</sup> (16) and Nck<sup>2flx/flx</sup> (5) mice were crossed to Lck-Cre mice (17) (a generous gift of Dr. J. Marth, University of California at San Diego, La Jolla, CA). HY-TCR (18), P14-TCR (19), and RAG2<sup>-/-</sup> mice were kindly provided by Drs. B. Rocha (INSERM), A. Freitas (Institut Pasteur, Paris, France), and S. Ezine (INSERM), respectively. Nck1<sup>-/-</sup>Nck<sup>2flx/flx</sup>Lck-Cre (Nck<sup>T<sup>+</sup></sup>) mice were back-crossed to C57BL/6 mice three to four times and crossed to RAG2<sup>-/-</sup> or P14-Nck.T<sup>-/-</sup> mutant mice. Given the high number of mutations (3–5) and the difficulty to obtain mutants and wild-type mice in the same litter, an initial set of experiments was conducted to establish the equivalence of Lck-Cre<sup>No</sup>Nck1<sup>-/-</sup>Nck<sup>2flx/flx</sup> littersmates, obtained from Lck-Cre<sup>+</sup>Nck1<sup>+</sup>Nck<sup>2flx/flx</sup> intercrossing, and Lck-Cre<sup>-</sup>Nck1<sup>+</sup>Nck<sup>2flx</sup> mice, obtained from Lck-Cre<sup>-</sup>Nck1<sup>+</sup>Nck2<sup>+/+</sup> intercrossing (Supplemental Fig. 1). Notably, Nck<sup>T<sup>+</sup></sup>- and Nck<sup>T<sup>+</sup></sup> mice on the C57BL/6.N10 background retained their phenotypic differences.

**Flow cytometry**

The Abs used are listed in Supplemental Table I. Surface staining was carried out according to standard procedures. DN thymocytes were defined as CD45.2<sup>+</sup>L<sup>-</sup>Lin<sup>-</sup> (NK1.1<sup>-</sup>Gr1<sup>-</sup>CD11b<sup>-</sup>CD11c<sup>-</sup>CD19<sup>+</sup>Ter119<sup>-</sup>) thymocytes. For CD3ε intracellular staining, Cytofix/Cytoperm and Perm/Wash Buffer (BD Biosciences, Franklin Lakes, NJ) were used according to the manufacturer’s instructions. Four-color FACS samples were acquired on a FACSCalibur (BD Biosciences); 8-color FACS samples were acquired on a BD FACSAria III (BD Biosciences Canto, using the BD FACS Diva acquisition software. Data analysis was performed using the FlowJo software (Tree Star, Ashland, OR).

**In vitro thymocyte stimulation**

Thymocytes from Nck<sup>T<sup>+</sup></sup>- and Nck<sup>T<sup>+</sup></sup> mice (4 × 10<sup>6</sup>/well) were cocultured with irradiated (30 Gy) T cell-depleted spleen cells from C57BL/6 mice (4 × 10<sup>5</sup>/well) in the presence of graded doses of the *Staphylococcus aureus* enterotoxin A (SEA). Alternatively, thymocytes (10<sup>5</sup>/well) were cultured in the presence of graded doses of plate-bound anti-CD3ε and anti-CD28 Abs. After 18 h of culture in a humidified 37°C, 5% CO<sub>2</sub> incubator, cells were harvested, stained for extracellular markers, and subsequently incubated with annexin V/propidium iodide, according to standard procedures. The amount of specific apoptosis was calculated by the following equation: (CD3/CD28-induced apoptosis – spontaneous apoptosis)/100 – spontaneous apoptosis)%.

**RT-PCR analysis**

Total RNA was extracted with the RNeasy kit (Qiagen, Hilden, Germany) and reverse transcribed with the ImProm-II Reverse Transcription System kit (Promega, Madison, WI) according to the manufacturer’s instructions. GAPDH amplification of graded doses of each cDNA sample was used to quantify and “normalize” their levels, using the ImageG software. After normalization, equal cDNA amounts were amplified using Nck1- and Nck2-specific primers.

**Bone marrow chimeras**

Bone marrow (BM) cells were obtained by flushing femurs and tibias of donor mice. After magnetic T cell depletion, 12 × 10<sup>6</sup> to 13 × 10<sup>6</sup> BM cells were injected i.v. into irradiated (4.5 Gy) RAG2<sup>-/-</sup> recipients. Mice were treated with antibiotic for 8 d, starting one day before BM transfer. BM chimeras were analyzed 6–8 wk after transfer.

**Immunoprecipitation and Western blot analysis**

Freshly isolated thymocytes were incubated with biotinylated α-CD3ε and α-CD8α (10 μg/ml) for 15 min on ice. After washing with cold medium, cells were prewarmed for 1 min at 37°C and cross-linked with prewarmed streptavidin (20 μg/ml). Lysis was performed in cold buffer containing 0.5% Nonidet P-40, 150 mM NaCl, 20 mM Tris-HCl (pH 7.6), 1 mM EDTA, 1 mM EGTA, 1 mM β-glycerophosphate freshly supplemented with protease inhibitors (Roche, Mannheim, Germany), 1 mM NaF, 1 mM Na<sub>3</sub>VO<sub>4</sub>, and 1 mM okadaic acid. The cytosolic fraction was assessed for protein content (BCA protein assay kit; Pierce, Rockford, IL). Equal protein amounts were immunoprecipitated with the indicated Abs. For subsequent Western blotting, immunoprecipitates were washed three times with lysis buffer. Immune complexes were resolved by SDS-PAGE, transferred to Immobilon-P (Millipore, Billerica, MA), and probed with biotinylated primary Abs, followed by streptavidin-HRP (Millipore). Whole-cell lysates resolved by SDS-PAGE were immunoblotted with Abs, including anti-phospho-linker of activated T cells (LAT) (Tyr191), and anti–phospho-ERK (Thr202/Tyr204, E10; Cell Signaling, Danvers, MA). Detection was carried out by chemiluminescence (Pierce, Rockford, IL). Membranes were stripped and reprobed for β-actin (AC-15; Sigma, St. Louis, MO) or ERK1/2 (rabbit polyclonal Ab; Cell Signaling).

**Statistics**

All two-group comparisons were performed with the exact Wilcoxon rank sum test using SAS version 9.1 (SAS Institute, Cary, NC). In the graphs of Figs. 1, 2, and 3, *p < 0.05 (significant) and **p < 0.001 (highly significant).

**Results**

**Targeted deletion of the Nck adapters in developing and mature thymocytes**

Deletion of both Nck adapters in the T cell compartment was obtained using a conditional genetic approach. Nck1<sup>-/-</sup> (16) and Nck<sup>2flx/flx</sup> (5) mice were crossed to Lck-Cre transgenic mice (17) to obtain Lck-Cre × Nck1<sup>-/-</sup>Nck<sup>2flx/flx</sup> mice, which will be referred to as Nck.T<sup>-/-</sup> throughout the paper, and Lck-Cre × Nck1<sup>+/+</sup>Nck2<sup>+/+</sup> controls, which will be referred to as Nck.T<sup>+</sup> mice (Supplemental Fig. 1). The expression of the Cre recombinase (Supplemental Fig. 2) and the deletion efficiency of the floxed Nck2 alleles (Supplemental Fig. 3) were analyzed at different stages of T cell development. Cre expression was low in immature thymocytes (DN1/DN2) and increased throughout thymic ontogeny to reach virtually 100% in mature thymocytes. At the mRNA level, expression of Nck2 was readily identified at the DN stage, detectable at low levels in DP thymocytes, and absent in mature single positive (SP) thymocytes (Supplemental Fig. 3A). At the protein level, the Nck proteins were undetectable in the thymic lyses of Nck.T<sup>-/-</sup> mice (Supplemental Fig. 3B), indicating that the residual expression of Nck2 was minimal and/or restricted to a minute thymocyte fraction.

**Reduced thymic cellularity in Nck.T<sup>-/-</sup> mice**

The phenotypic analysis of Nck.T<sup>-/-</sup> mice revealed a 3- to 4-fold reduction of the thymic cellularity (Fig. 1A) compared with that of age-matched Nck.T<sup>+</sup> mice. The thymic architecture and the levels of in vivo apoptotic cells remained unaffected by Nck deletion, as assessed by histological analysis and TUNEL assay (Supplemental Fig. 4). The decreased thymic cellularity in Nck.T<sup>-/-</sup> mice was mainly accounted for by a reduction in DP cells (3- to 4-fold) in the absence of significant alterations of the CD4/CD8 profile (Fig. 1C). Notably, DN thymocytes were less severely

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The transition from the DN3a to DN3b stage (21). In Nck.T
arrangement (20). CD27 is upregulated after TCR
-independent experiments. Shaded histograms represent isotype controls.

increased in Nck-deficient DP thymocytes (p < 0.001). The number of CD8low
TCRlow ISP thymocytes was significantly reduced in Nck.T
mice compared with those of Nck.T+/+ mice. *p < 0.05; **p < 0.001. B. The CD4+CD8
profile remained unaltered in the absence of Nck. D and E. The percentage of Lin
CD45.2+CD4+CD8+CD25+CD44+ (DN3) thymocytes was increased in Nck.T
mice compared with that of Nck.T+/+ controls and was characterized by a decrease in CD27 expression. F. Expression of TCRβ, CD5, and CD69 on DP (top) and SP8 (bottom) thymocytes from Nck.T
(dotted line, n = 6) and Nck.T+/+ controls (solid line, n = 5). G. The surface levels of CD3e
were increased in Nck-deficient DP thymocytes (top) in the absence of alterations of the total cellular pool (bottom). All data are representative of three to five independent experiments. Shaded histograms represent isotype controls.

Reduced thymic cellularity and partial block at the β-checkpoint in Nck.T
mice. A. The thymic cellularity was reduced in 4- to 5-wk-old
Nck.T
mice (△, n = 13) compared with that of Nck.T+/+ controls (□, n = 14, p < 0.00001). CD44+CD8α (DP; p < 0.00001) and CD4+CD8α (SP4; p < 0.001) thymocytes were strongly reduced in Nck.T
mice compared with those of Nck.T+/+ mice. *p < 0.05; **p < 0.001. B. The number of CD8low
TCRlow ISP thymocytes was significantly reduced in Nck.T
mice compared with that of wild-type controls (p = 0.0001). **p < 0.001. C. The CD4+CD8
profile remained unaltered in the absence of Nck. D and E. The percentage of Lin
CD45.2+CD4+CD8+CD25+CD44+ (DN3) thymocytes was increased in Nck.T
mice compared with that of Nck.T+/+ controls and was characterized by a decrease in CD27 expression. F. Expression of TCRβ, CD5, and CD69 on DP (top) and SP8 (bottom) thymocytes from Nck.T
(dotted line, n = 6) and Nck.T+/+ controls (solid line, n = 5). G. The surface levels of CD3e
were increased in Nck-deficient DP thymocytes (top) in the absence of alterations of the total cellular pool (bottom). All data are representative of three to five independent experiments. Shaded histograms represent isotype controls.

reduced (1.5- to 3-fold), suggesting potential alterations in the
DN-DP transition. Phenotypically, the expression of CD44 and
CD25 on Lin+CD45.2+DN thymocytes (Supplemental Fig. 5)
defines four developmental stages CD25−CD44+ (DN1), CD25+CD44+ (DN2), CD25+CD44−(DN3), and CD25−CD44−(DN4), which correspond with the phases of TCRβ gene rearrangement (20). CD27 is upregulated after TCRβ selection, in the transition from the DN3a to DN3b stage (21). In Nck.T−/− mice, we observed an increase in Lin−CD45.2+CD25+CD44−(DN3) thymocytes (Fig. 1D) associated with a reduction in post-
β selection CD27high cells (Fig. 1E). As maturation to the DP
stage is associated with CD8 upregulation and thymocyte pro-
iferation, we next investigated the characteristics of CD8low
TCRlow immature single-positive (ISP) thymocytes (Supplemental Fig. 6). Total CD8low thymocytes (Supplemental Fig. 6C) and ISP thymocytes (Fig. 1B) were significantly reduced in Nck.
T−/− mice. However, the intrinsic proliferative capacity of ISP
thymocytes, similar to that of other thymic subsets, was not af-
fected by Nck deletion (Supplemental Fig. 7). Thus, our findings
are compatible with the hypothesis that the Nck adapters influence
TCRβ selection and the progression to the DP stage.

Nck deletion results in TCR overexpression on DP thymocytes

The Nck adapters have been implicated in the regulation of the
surface levels of the TCR (11, 22, 23). The levels of TCR
expression are finely regulated at the DP stage (24), as both positive
and negative selection depend on the strength of TCR signaling
(25). Notably, we found that the surface levels of TCRβ (Fig. 1F,
top) and CD3e (Fig. 1G, top) expression were increased in DP
cells from Nck.T−/− mice compared with those of wild-type
controls. However, loss of Nck function did not alter the size of the
total CD3e pool in DP thymocytes, as assessed by intracellular
staining (Fig. 1G, bottom). Upregulation of CD5 and CD69
has been linked to positive selection (26). Notably, both markers
were prematurely increased on Nck-deficient DP thymocytes (Fig. 1F,
top). However, no obvious phenotypic alterations were found in
mature SP thymocytes from Nck.T−/− mice (Fig. 1F, bottom), which also exhibited comparable Vβ-chain frequency and distribu-
tion to Nck.T+/+ thymocytes (Supplemental Fig. 8). Taken to-
gether, the phenotypic association of altered TCR and CD5
expression on Nck-deficient DP thymocytes points toward a po-
tential dysregulation in TCR signal strength with possible effects
on thymic selection.

The Nck adapters are required for deletion of thymocytes
expressing a “weak” TCR

To assess the impact of Nck on thymocyte selection, we used the
HY-TCR transgenic model. HY-TCR T cells recognize a peptide
derived from the male minor histocompatibility Ag HY in the
context of H-2Dβ (18). The presence of the target Ag in the thymi
of male mice results in HY-TCR thymocyte deletion. If, as in the
current study, the transgenic HY–TCRα-chain and HY–TCRβ-
chain are concomitantly expressed at the DN stage, negative
selection occurs in the thymic cortex, prior to the DP stage (18). In

![Figure 1](http://www.jimmunol.org/DownloadedFrom/170616/1035657013)

**FIGURE 1.** Reduced thymic cellularity and partial block at the β-checkpoint in Nck.T−/− mice. A. The thymic cellularity was reduced in 4- to 5-wk-old Nck.T−/− mice (△, n = 13) compared with that of Nck.T+/+ controls (□, n = 14, p < 0.00001). CD44+CD8α (DP; p < 0.00001) and CD4+CD8α (SP4; p < 0.001) thymocytes were strongly reduced in Nck.T−/− mice compared with those of Nck.T+/+ mice. *p < 0.05; **p < 0.001. B. The number of CD8low TCRlow ISP thymocytes was significantly reduced in Nck.T−/− mice compared with that of wild-type controls (p = 0.0001). **p < 0.001. C. The CD4+CD8 profile remained unaltered in the absence of Nck. D and E. The percentage of Lin−CD45.2+CD4+CD8+CD25+CD44+ (DN3) thymocytes was increased in Nck.
T−/− mice compared with that of Nck.T+/+ controls and was characterized by a decrease in CD27 expression. F. Expression of TCRβ, CD5, and CD69 on DP (top) and SP8 (bottom) thymocytes from Nck.T−/− (dotted line, n = 6) and Nck.T+/+ controls (solid line, n = 5). G. The surface levels of CD3e were increased in Nck-deficient DP thymocytes (top) in the absence of alterations of the total cellular pool (bottom). All data are representative of three to five independent experiments. Shaded histograms represent isotype controls.
contrast, if expression of the transgenic TCRα-chain is more physiologically induced at the DP stage, HY-TCR T cells are eliminated at the single-positive or medullary stage (27). One of the key features of the HY-TCR is its relatively low affinity for the HY peptide–H2-Db complex, which is responsible for its exquisite sensitivity to alterations of the TCR signal strength (28). The “weakness” of the HY-TCR leads to inefficient deletion and peripheral “leakage” of HY-TCR T cells, which remain anergic and express reduced levels of the CD8αβ coreceptor. Unlike non-TCR-transgenic Nck.T−/− mice, HY-TCR RAG−/−Nck.T−/− males exhibited a thymic cellularity comparable with that of wild-type mice (Supplemental Fig. 9A). Their CD4/CD8 profile was characterized by a marked increase in DP thymocytes (Fig. 2A). In the periphery, Nck-deficient HY-TCR T cells were reduced (Fig. 2B) and exhibited increased surface levels of the CD8αβ coreceptor (Fig. 2C, left). Notably, the increase in CD8 expression on Nck-deficient mature T cells never exceeded the maximal levels of male wild-type T cells, remaining lower than that of HY-TCR T cells in female mice (Fig. 2C, right). Taken together, these alterations point toward a crucial role for the Nck adapters in shaping the selection and phenotypic profile of HY-TCR T cells. To better understand the role of the Nck adapters in the selection of the polyclonal repertoire, thymocytes from Nck.T−/− and Nck.T+/+ mice were stimulated in vitro with graded doses of SEA or of CD3/CD28. Both treatments induce apoptosis of DP thymocytes upon TCR engagement, which, in the case of SEA, is selective for thymocytes expressing Vβ11 (29). Apoptosis of Nck-deficient Vβ11+ DP thymocytes was less efficient than that of Nck.T+/+ thymocytes at low but not high doses of SEA (Fig. 2D, bottom). No deletion was observed in the case of DP thymocytes expressing Vβ8.1/2 (Fig. 2D, top). Similarly, Nck deletion conferred increased resistance to the induction of apoptosis by low doses of CD3/CD28 but did not alter the response to high doses of CD3/CD28 (Fig. 2E), as assessed by annexin V/propidium iodide staining. Taken together, these findings indicate that in the absence of Nck, DP thymocytes lose sensitivity to weak TCR-mediated stimulation and suggest that Nck is required for thymocyte deletion by low-affinity ligands.

**Impaired negative selection does not trigger spontaneous autoimmunity in Nck.T−/− mice**

We next investigated whether HY-TCR T cells that had escaped negative selection in HY-TCR RAG−/−Nck.T−/− males induced spontaneous autoimmunity. HY-TCR RAG−/−Nck.T−/− males did not lose weight (Supplemental Fig. 9B) or exhibit any signs of running syndrome. Histologically, there were no signs of immune

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**FIGURE 2.** The Nck adapters are essential for negative selection of HY-TCR T cells. A, The percentage of DP thymocytes was markedly increased in HY-TCR RAG−/−Nck.T−/− males (right, n = 5) compared with that of HY-TCR RAG−/−Nck.T+/+ controls (left, n = 5). B, In the spleen, the percentage of HY-TCR T cells, as assessed by staining with the transgenic TCRα (T3.70) and TCRβ (Vβ8.1/2)-chains, was reduced in HY-TCR RAG−/−Nck.T−/− males compared with that of wild-type controls. C, In HY-TCR RAG−/−Nck.T−/− males, HY-TCR T cells exhibited a less profound downregulation of the CD8αβ coreceptor than that in HY-TCR RAG−/−Nck.T+/+ males. The expression of CD8αβ in HY-TCR RAG−/−Nck.T−/− females (right) is shown for comparison. D, Upon in vitro stimulation with graded doses of SEA, thymocytes from Nck.T−/− and Nck.T+/+ mice were harvested and stained with Abs specific for CD4, CD8, Vβ11 (bottom), or Vβ8.1/2 (top). SEA induces apoptosis of thymocytes expressing Vβ11 but has no effect on thymocytes expressing Vβ8.1/2. Deletion of Vβ11+ DP thymocytes from Nck.T−/− mice was less efficient than deletion of those from Nck.T+/+ mice at low doses of SEA (0.1 μg/ml, p = 0.049; 0.01 μg/ml, p = 0.049). In contrast, no differences were found in the levels of Vβ11+ deletion at high SEA concentrations (10 μg/ml, p = 0.079). *p < 0.05. The levels of Vβ8.1/2+ DP thymocytes remained unaltered upon exposure to SEA. Cumulative data from four independent experiments are shown. E, Thymocytes from Nck.T−/− (n = 6) and Nck.T+/+ (n = 7) mice were stimulated with graded doses of plate-bound CD3/CD28 and analyzed by flow cytometry after 18 h of culture. As assessed by annexin V/propidium iodide staining, the levels of specific apoptosis were reduced in DP thymocytes from Nck.T−/− mice at low (p = 0.0066) but not high doses of CD3/CD28. *p < 0.05. Cumulative data from three independent experiments are shown.
infiltration or tissue damage in the digestive tract, lungs, kidneys, muscles, brain, or testes of HY-TCR RAG−/− Nck.T−/− males (data not shown). However, a mild perivascular infiltration in the absence of tissue damage was found in the liver of HY-TCR RAG−/− Nck.T−/− mice, irrespective of their gender (Supplemental Fig. 9C, 9D). The similarity of the liver infiltrate in male and female HY-TCR RAG−/−Nck.T−/− mice and its benign and nondestructive characteristics argue against its autoimmune origin. Thus, our findings rule out the hypothesis that Nck deletion interferes with self-tolerance in HY-TCR RAG−/−Nck.T−/− males.

Nck is required for positive selection of low-avidity HY-TCR T cells

In HY-TCR transgenic females, the absence of HY expression allows maturation of HY-TCR T cells. In comparison with wild-type mice, HY-TCR RAG−/−Nck.T−/− females exhibited a 3- to 5-fold reduction in the percentage of mature SP8 thymocytes (Fig. 3A), associated with TCR upregulation on DP thymocytes (Fig. 3B), and a reduction in the absolute number of splenic HY-TCR T cells (Fig. 3C). Thus, Nck is required for positive selection of HY-TCR T cells.

We reasoned that if the HY-TCR had become “unfit” for positive selection in the absence of Nck, rearrangement of endogenous TCRα-chains may produce thymocytes with higher TCR avidity for endogenous ligands and better chances for positive selection. To test this hypothesis, we used RAG-sufficient HY-TCR Nck.T−/− and HY-TCR Nck.T+/+ females and compared the expression of the transgenic TCRα (TCRαT) and TCRβ (TCRβT) chains on mature SP8 thymocytes. Because the TCRα-chain and TCRβ-chain form a heterodimer, selective downregulation of TCRαT without significant alterations of TCRβT expression indicates TCRαT pairing with an endogenously rearranged TCRα-chain (TCRαE). Our findings indicate a marked reduction in mature HY-TCR SP8 cells in the absence of Nck (Fig. 3D and Supplemental Fig. 10A, 10B) and a relative increase in the percentage of SP8 thymocytes expressing endogenously rearranged TCRα-chains (Fig. 3E and Supplemental Fig. 10A). Taken together, our data are compatible with the hypothesis that Nck deletion results in a reduction in the intrinsic “fitness” of the HY-TCR for positive selection, partially compensated by receptor editing.

The thymic defects of HY-TCR Nck.T−/− mice are intrinsic to hematopoietic cells

We next sought to define the cellular origin of the selection defects observed in Nck.T−/− mice. Because Nck/1 is systemically deleted and potential alterations of thymic epithelial cells may alter thymic selection, we generated BM chimeras in sex-matched RAG2−/− recipients. The phenotypic analysis of the chimeras revealed that their phenotype faithfully reproduced the defects of negative (Fig. 4A) and positive (Fig. 4B) selection observed in the HY-TCR RAG−/−Nck.T−/− donors. Thus, the thymic selection defects observed in HY-TCR Nck.T−/− mice are due to Nck-dependent alterations intrinsic to hematopoietic cells.

Nck is dispensable for the selection of high-avidity T cells

Several lines of evidence suggest that the T cells have different sensitivity to mutations of the TCR signaling apparatus (28), as a function of their avidity for the selecting of self-pMHC complexes. Thus, we investigated the impact of Nck deletion on the ontogeny of high-avidity P14-TCR T cells (19). The thymic cellularity and the CD4/CD8 profile of P14-TCR Nck.T−/− mice remained unaffected by in vivo Nck deletion (Fig. 5A). Notably, the early phases of T cell development were impaired in P14-TCR Nck.T−/− mice, as suggested by the increase in the Lin−CD45.2+ CD44+ CD25+ DN3 fraction (Fig. 5B) and by the relative decrease in the CD27hi DN3 fraction (Fig. 5C). Similar to the non-transgenic and HY-TCR models, the levels of TCR expression were increased on DP cells from P14-TCR Nck.T−/− mice (Fig. 5D) but were comparable with those of wild-type mice in the SP8 fraction (data not shown). Within the SP8 mature thymocyte fraction, the percentage of cells expressing endogenously rearranged TCRα-chains was not increased in the absence of Nck (Fig. 5E). Taken together, these findings indicate that Nck is not required for positive selection of high-avidity T cells.

Reduced ERK phosphorylation in Nck-deficient thymocytes

The CD3ε.PRS is reportedly accessible to Nck upon a conformational change induced by TCR engagement (10). The APA1/1 mAb specifically recognizes the CD3ε.PRS and has been used to detect the CD3ε conformer accessible to Nck binding (30). Freshly isolated thymocytes revealed a low but significant level of APA1/1

*FIGURE 3. Requirement of Nck for positive selection of low-avidity HY-TCR T cells. A, The percentage of SP8 cells was strongly reduced in HY-TCR RAG−/− Nck.T−/− females (right, n = 3) compared with that of HY-TCR RAG−/− Nck.T+/− (left, n = 2), indicating impaired positive selection. B, Expression of the transgenic TCRα (T3.70) and TCRβ (Vβ8.1/2)-chains was increased on Nck.T−/− DP thymocytes (dotted line) compared with that of Nck.T+/− DP cells (solid line). C, The number of splenic CD8+ HY-TCR T cells was markedly reduced in HY-TCR RAG−/− Nck.T−/− females (n = 7) compared with that of wild-type controls (n = 6; p = 0.0135). D*, p < 0.05. D, The percentage of SP8 thymocytes was lower in HY-TCR RAG−/− Nck.T−/− females (n = 5) compared with that of HY-TCR RAG−/− Nck.T+/− females (n = 5). E, Association of the transgenic TCRβ-chain with endogenously rearranged TCRα-chains is associated with downregulation of the transgenic TCRα (T3.70)-chain. HY-TCR RAG−/− Nck.T−/− females were characterized by an increased percentage of SP8 cells expressing low levels of the transgenic TCRα-chain. Percentages and absolute numbers of SP8, SP8 TCRαβ, and TCRαβ are shown in Supplemental Fig. 10.
Nck IN T CELL DEVELOPMENT

Discussion

The current study provides genetic evidence for a crucial role of the Nck adapters in T cell ontogeny using the combination of a conditional genetic system with two TCR transgenic models. Nck deletion resulted in a series of phenotypic and functional alterations including 1) a relative increase in DN3 thymocytes; 2) TCR over-expression on DP thymocytes; and 3) impairment of positive and negative selection by ligands of low antigenic potency.

In both TCR transgenic and non-transgenic models analyzed in this study, the percentage of DN3 thymocytes was increased in the absence of Nck, suggesting the involvement of these adapters in TCRβ-selection. Further analysis revealed a relative decline in the CD27high DN3 fraction and a drastic reduction in ISP thymocytes, which point toward a role for Nck in TCRβ selection. Additional alterations induced by Nck in the early phases of T cell development may not be apparent in our conditional genetic model because Nck is not required for CD3ε.PRS “exposure.” The earliest step after TCR engagement is the activation of Src protein tyrosine kinases (Lck and Fyn), leading to phosphorylation of the CD3 ITAMs. Recruitment of ZAP-70 follows, leading to a cascade of events, including phosphorylation of the LAT adapter (31). Nck deletion was associated with an apparent increase in CD3ε phosphorylation under resting and stimulating conditions (Supplemental Fig. 13A). Upon TCR ligation, binding of diacylglycerol to the Ras activators RasGRP (31) leads to activation of the Ras/MAPK pathway, which is essential for thymic selection (2). Activated thymocytes from Nck.T−/− mice exhibited lower levels of ERK1/2 phosphorylation compared with those of Nck.T+/+ controls, as assessed by immunoblotting with phospho-specific Abs recognizing the active forms of ERK1 and ERK2 (Fig. 6B and Supplemental Fig. 13B, 13C). Intracellular staining also revealed a reduction in the levels of ERK1/2 phosphorylation, which was particularly pronounced in mature SP8 cells (Fig. 6C). Taken together, our data suggest the involvement of the Nck adapters at multiple levels of the TCR signaling cascade and indicate their essential role in ERK1/2 activation.

**FIGURE 4.** Hematopoietic origin of the thymic defects of HY-TCR Nck.T−/− mice. BM chimeras were generated by injection of BM from HY-TCR RAG−/− (HYRAG) Nck.T+/+ or Nck.T−/− mice into irradiated (4.5 Gy) female BM chimeras exhibited a reduction in the percentage of SP8, similar to their BM donors (Nck.T+/+, n = 13, top; Nck.T−/−, n = 10, bottom).

**FIGURE 5.** Nck is not required for the selection of high-avidity T cells. A, P14-TCR.Nck.T−/− mice (n = 9) had a CD4/CD8 profile comparable with that of P14.Nck.T+/+ controls (n = 9). B, Nck deletion resulted in a relative increase in the percentage of Lin−CD45.2+/CD4−CD8+CD25−CD44− DN3 thymocytes compared with that of wild-type controls. C, Upregulation of CD27 is associated with the transition from DN3 to DN3b upon TCRβ selection (21). Nck deletion was associated with a relative decrease in the CD27high DN3 fraction. D, Nck-deficient DP thymocytes from P14-TCR.Nck.T−/− mice were characterized by enhanced surface expression of the TCR. E, The expression of endogenously rearranged TCRα-chains in mature SP8 thymocytes was assessed by Abs specific for Vα2 and Vβ8.1/2, respectively recognizing the P14–TCRα-chain and P14–TCRβ-chain. The percentage of the transgenic TCRα-chain and TCRβ-chain was comparable in the presence/absence of Nck. Data are representative of at least three independent experiments.
of the suboptimal deletion of the Nck2flox alleles in DN thymocytes. Notably, a partial block at the DN3 stage is shared by other dele-

Loss of Nck function resulted in profound phenotypic alter-

The selective impairment of low-avidity TCR selection is

The altered ERK activation observed in the absence of Nck is

The selective impairment of low-avidity TCR selection is reminiscent of the phenotype of CD3ε.ITAM (43), TCRζ.ITAMs (28, 44). To this end, it is interesting that CD3ε phosphorylation is increased in Nck-deficient thymocytes under both resting and stimulat-

Indeed, one of the key observations of our study is that the Nck adapters are selectively required for positive selection of low-

FIGURE 6. Altered signaling in Nck-deficient thymocytes. A, LAT phos-

The increased expression of the CD8αβ coreceptor on HY-TCR T cells from transgenic males may fall into a state of hyporesponsiveness, similar to that described in the polyclonal repertoire (15).

The selective impairment of low-avidity TCR selection is reminiscent of the phenotype of CD3ε.ITAM (43), TCRζ.ITAMs (28, 44). To this end, it is interesting that CD3ε phosphorylation is increased in Nck-deficient thymocytes under both resting and stimulating conditions. As the total CD3ε cell pool was not affected by Nck deletion, such effect is unlikely to be induced by CD3ε overexpression on Nck-deficient DP thymocytes. Notably, it has been hypothesized that the Nck-binding CD3ε.PRS PxxDY170 region (11, 45) may work as a molecular switch, binding to SH3 domain-containing proteins, such as Nck, under resting conditions and to SH2 domain-containing proteins upon activation-induced phosphorylation (46). In this scenario, loss of Nck function may further perturb the phosphorylation state of CD3ε, thereby altering the threshold of TCR responsiveness.

The altered ERK activation observed in the absence of Nck is likely to play a crucial role in the phenotype of Nck.Tε/PΔ mice, given the importance of ERK phosphorylation in positive and negative selection (2, 25). As a reduction in TCR-mediated ERK phosphorylation is a hallmark of clonal anergy (47), the strong reduction of ERK phosphorylation in SP thymocytes and peripheral T cells from Nck.Tε/PΔ mice (27) raises the question of the...
potential link between Nck-deficient T cell hyporesponsiveness and anergy. Notably, a reduction in ERK phosphorylation was not observed in CD3e.PRS knock-in mice (13) but was present in WASP 


Supplementary Figure 1. Phenotypic characterization of Nck.T<sup>T/+</sup> control mice. Loss of *in vivo* Nck function in developing and mature T cells was obtained with a conditional genetic approach. Nck1<sup>+/−</sup> (16) and Nck2<sup>flx/flx</sup> (5) mice were crossed to *Lck*-Cre transgenic mice (17) to obtain *Lck*-Cre x Nck1<sup>+/−</sup>Nck2<sup>flx/flx</sup> mice, which are referred to as Nck.T<sup>T−</sup> mice. As the presence of 3-5 mutations strongly reduced the probability of obtaining mutants and wild type mice in the same litter, preliminary experiments were conducted to establish the equivalence of *Lck*-Cre<sup>tg</sup> Nck1<sup>+/+</sup>Nck2<sup>+/+</sup> (Cre) mice, obtained from *Lck*-Cre<sup>tg</sup> Nck1<sup>+/−</sup>Nck2<sup>flx/+</sup> intercrossing, and *Lck*-Cre<sup>Ntg</sup> Nck1<sup>+/−</sup>Nck2<sup>flx/flx</sup> (Non-Cre) littermates, obtained from *Lck*-Cre<sup>het</sup> Nck1<sup>+/−</sup>Nck2<sup>flx/flx</sup> intercrossing. The analysis of Cre-expressing and non-expressing control Nck.T<sup>T+/+</sup> mice revealed overlapping phenotypic characteristics. Notably, the CD4/CD8 profile of total thymocytes (A), the CD44/CD25 profile of Lin−CD45.2<sup>+</sup>CD4<sup>−</sup>CD8<sup>−</sup> DN thymocytes (B) and the levels of CD3ε expression on DP and SP thymocytes (C) were indistinguishable in the presence/absence of Cre expression. Results are representative of three independent experiments. Control mice are referred to as Nck.T<sup>T+/+</sup> throughout the text.

Supplementary Figure 2. Lck-driven Cre recombinase expression in Nck.T<sup>T−</sup> mice.

Targeted Nck deletion to developing and mature T cells was obtained by crossing Nck1<sup>−/−</sup>Nck2<sup>flx/flx</sup> mice with *Lck*-Cre transgenic mice. Cre recombinase expression was assessed in the thymus by flow cytometry. Cre expression, already detectable at the CD4<sup>−</sup>CD8<sup>−</sup> DN1/2 stage, progressively increased throughout thymic development to reach virtually 100% expression starting at the DP stage. Cre expression was comparable in Nck.T<sup>T−</sup> (N = 9) and Nck.T<sup>T+/+</sup> (N = 7) mice. Data are representative of five independent experiments.
Supplementary Figure 3. Efficient Nck deletion in Nck.T^{-/-} mice.

(A). To assess the deletion efficiency of the Nck2.floxed allele, Nck2 expression was assessed by RT-PCR on different lymphocyte subsets, purified by cell sorting (FACSAria; BD; purity = 97.2 ± 1%) from the thymus (Panel A, CD4^+CD8^- (DN), CD4^-CD8^- (DP), CD4^-CD8^+ (SP8) and CD4^+CD8^- (SP4) thymocytes) of various experimental mouse strains. Cells were derived from Nck.T^{-/-} mice (-/-, N=2) and compared to those from Nck.T^{+/+} (+/+, N=2), Lck-Cre Nck1^{+/+} Nck2^{flx/flx} (flx/Cre, N=2) and Nck1^{+/+} Nck2^{flx/flx} (flx/flx, N=2) mice. Nck2 expression was readily detectable in DN thymocytes of Nck.T^{-/-} mice, as a result of the inefficient expression of the Cre recombinase at very early stages of T cell development (DN1/2, Suppl. Fig.1A). Nck2 expression was very low in DP thymocytes, undetectable in mature thymocytes (SP4 and SP8). Data are representative of two independent experiments. (B) At the protein level, Nck was not detectable in thymic lysates from Nck.T^{-/-} mice, as assessed by immunoblotting with an antibody recognizing both Nck1 and Nck2 (NC-20, Abcam). Data are representative of three separate experiments.

Supplementary Fig. 4: Nck deletion does not alter in vivo thymic apoptosis.

The role of the Nck adaptors in thymocyte apoptosis was assessed by TUNEL assay on cryosections of thymi from age and sex-matched Nck.T^{-/-} (N = 6) and Nck.T^{+/+} (N = 7) mice. The architectural organisation of the thymi from mutant and wild type mice was comparable and no differences in the number or location of apoptotic cells were identified.

Supplementary Fig. 5: Gating strategy for DN thymocytes. The analysis of the DN thymocyte subsets was carried out as follows. In brief, after gating on live thymocytes by FSC/SSC dead cell exclusion (left), a dot plot defined cells positive for CD45.2 (104-PE.Cy7) and negative for markers specific of the erythrocyte (Ter119-PerCP.Cy5), granulocyte (Gr1-
PerCP.Cy5), B lymphocyte (1D3-PerCP.Cy5), macrophage (M1/70-PerCP.Cy5), dedritic cell (HL3-PerCP.Cy5) and NK cell (PK136-PerCP.Cy5) lineages. Lin^CD45.2^ cells were subsequently examined for expression of CD4 (RM4-5-Quantum Dot605) and CD8 (53-6.7-FITC). Gated CD4^-CD8^- DN thymocytes were then examined for expression (right) of CD44 (IM7-PE.Cy7) and CD25 (PC61-APC).

Supplementary Fig. 6: Reduction of the CD8^{low} TCR^{low} thymocyte fraction in the absence of Nck. Thymocytes which have undergone TCRβ-selection progress through a developmental phase characterised by upregulation of the CD8 coreceptor and several rounds of proliferation. (A) In the thymus, CD8^{low} cells comprise immature single positive thymocytes (ISP) progressing from the DN to the DP stage, as well as thymocytes which, having undergone positive selection, progress to the SP8 stage. (B) Immature CD8^{low} thymocytes (ISP) are characterised by low TCR expression, while mature CD8^{low} thymocytes have high TCR expression. (C). The total number of CD8^{low} thymocytes was significantly reduced in Nck.T^-/- mice, when compared to age-matched wild type controls (p < 0.0001, left). Notably, the immature CD8^{low} TCR^{low} ISP fraction was reduced in Nck.T^-/- mice, in which an inverted ratio between immature (TCR^{low}) and mature (TCR^{high}) thymocytes (C; p=0.0014, right) was found. Representative (B) and cumulative (C) data from five independent experiments are shown.

Supplementary Fig. 7: Nck deletion does not affect in vivo thymocyte proliferation. In vivo thymocyte proliferation was assessed by intra-peritoneal administration of BrdU to 4 week-old Nck.T^-/- and Nck.T^{+/+} mice. BrdU incorporation was measured 2 hours after injection by intracellular staining, using the Pharmingen BrdU Flow Kit (BD Biosciences), according to the manufacturer’s instructions. BrdU/DAPI staining defines different phases of the cell cycle,
namely G0/G1 \((\text{DAPI}^{\text{low}}\text{BrdU}^{\text{low}})\), S \((\text{DAPI}^{\text{int}}\text{BrdU}^{\text{high}})\) and G2/M \((\text{DAPI}^{\text{high}}\text{BrdU}^{\text{low}})\). The levels of BrdU incorporation in Nck.T\(^{-/-}\) \((N = 13)\) and Nck.T\(^{+/+}\) \((N = 13)\) mice were comparable in all subsets examined.

Supplementary Fig. 8: \textit{Nck deletion does not alter V\(\beta\) chain usage in Nck.T\(^{-/-}\) mice.}

Thymocytes from Nck.T\(^{-/-}\) \((N = 4)\) and Nck.T\(^{+/+}\) \((N = 4)\) mice were stained with antibodies specific for CD4, CD8 and individual TCR V\(\beta\) chains \((\text{BD Pharmingen kit})\), according to standard procedures. The cumulative percentages \((\text{mean} \pm \text{SEM})\) of SP8 \((\text{top})\) and SP4 \((\text{bottom})\) thymocytes expressing different V\(\beta\) chains in Nck.T\(^{-/-}\) and Nck.T\(^{+/+}\) mice are depicted. Loss of Nck function did not perturb V\(\beta\) chain usage in Nck.T\(^{-/-}\) mice. As naturally occurring superantigens are highly efficient in inducing thymocyte deletion, we hypothesize that their “avidity” may fall out of the range influenced by Nck \((\text{as exemplified in Fig. 2D})\), leaving unaltered the frequency of mature thymocytes expressing different V\(\beta\) chains. Data were obtained in three independent experiments.

Supplementary Fig. 9: \textit{Nck deletion does not induce spontaneous autoimmunity in HY-TCR RAG\(^{-/-}\) Nck.T\(^{-/-}\) males.} \((A)\) Total number of thymocyte was comparable in HY-TCR RAG\(^{-/-}\) Nck.T\(^{-/-}\) \((N = 8)\) and Nck.T\(^{+/+}\) \((N = 6)\) mice \((p = 0.282)\). \((B)\) 4-6 months-old HY-TCR RAG\(^{-/-}\) Nck.T\(^{-/-}\) \((N = 6)\) males did not display any signs of a “runting” syndrome and their body weight was comparable to that of age-matched HY-TCR RAG\(^{-/-}\) Nck.T\(^{+/+}\) males \((N = 5)\). \((C)\) A thorough histological analysis was performed on the stomach, small intestine, colon, cecum, lungs, kidneys, muscles, brain and testes of 4-6 months-old HY-TCR RAG\(^{-/-}\) Nck.T\(^{-/-}\) \((N = 6)\) and HY-TCR RAG\(^{-/-}\) Nck.T\(^{+/+}\) \((N = 5)\) mice. There were no signs of immune infiltration in any of these organs, irrespectively of the presence/absence of Nck \((\text{data not shown})\). However, livers from HY-TCR RAG\(^{-/-}\) Nck.T\(^{-/-}\) males \((\text{top})\) contained small,
scattered, non-destructive, mononuclear infiltrations of the portal fields and/or lobular parenchyma, which did not resemble the typical histopathology of graft-versus-host disease or autoimmune hepatitis. Surprisingly, the characteristic of the liver infiltrates were similar in HY-TCR RAG<sup>-/-</sup> Nck.T<sup>-/-</sup> males and females (<em>bottom</em>). Such similarity argues against an HY-driven autoimmune origin of the infiltrate. (<em>D</em>) Alanine aminotransferase (ALT) is an enzyme present in hepatocytes, and is released in the blood in case of liver damage. Its physiological plasma levels range between 9-60 IU/L. To assess whether liver infiltration in HY-TCR RAG<sup>-/-</sup> Nck.T<sup>-/-</sup> mice resulted in tissue damage, plasma ALT levels were assessed in HY-TCR RAG<sup>+/+</sup> Nck.T<sup>-/-</sup> males (N = 6) and females (N = 3), as well as in males (N = 6) and females (N = 7) wild type controls. Blood samples were assayed fresh, using Reflotron strips on a Reflotron Plus apparatus (Roche), according to the manufacturer’s instructions. A shaded grey line indicates the maximal physiological levels of plasma ALT. As a positive control for immune-mediated liver damage, we compared the ALT levels of HY-TCR RAG<sup>-/-</sup> Nck.T<sup>-/-</sup> mice to those of Nck.T<sup>+/+</sup> mice (N = 4) injected intravenously with Concanavalin A (20 mg/kg in 200 μl pyrogen-free saline) and assayed 14 hours after ConA injection.

**Supplementary Fig. 10:** Nck deletion results in a drastic reduction of SP8 thymocytes, which are enriched for expression of endogenously rearranged TCRα chains. We have quantified the percentage (A) and absolute numbers (B) of total HY-TCR SP8 thymocytes (<em>left</em>), SP8 expressing the transgenic TCRα and TCRβ chains (TCRα<sub>T</sub>β<sub>T</sub>, <em>middle</em>), or an endogenously rearranged TCRα chain together with the transgenic TCRβ chain (TCRα<sub>E</sub>β<sub>T</sub>, <em>right</em>) in HY-TCR RAG<sup>+/+</sup> Nck.T<sup>-/-</sup> (N = 6) and Nck.T<sup>+/+</sup> (N = 5) females. The percentage and absolute numbers of total SP8 were drastically reduced in the absence of Nck (p = 0.0009 and p < 0.0001, respectively). We observed only a 10-20% decrease in the percentage of SP8Vα<sub>T</sub>β<sub>T</sub>, which was extremely low, when compared to the strong decrease in
the percentage of peripheral CD8⁺ Vα1βT T cells (15). The decline in Nck-deficient SP8VαVβT was associated with a relative increase in the percentage SP8VαEβT (p = 0.0029). As a result of the alterations in the percentage of SP8VαVβT and SP8VαEβT, the absolute number of SP8VαVβT was drastically reduced (p < 0.0001) in HY-TCR RAG⁺/⁺ Nck.T⁻/⁻ females, while the absolute number of SP8VαEβT was comparable in the presence/absence of Nck (p=0.8131).

**Suppl. Fig. 11: Nck is not required for exposure of the CD3εPRS.** The APA1/1 antibody recognizes the proline reach sequence (PRS) of CD3ε, which is reportedly unmasked by TCR engagement (10). For APA1/1 staining, thymocytes were fixed with 2.5% formaldehyde (37°C, 10min) and permeabilized with 90% ice-cold methanol (30min). APA1/1 Ab (Upstate) staining was performed prior to staining with extra-cellular markers. Thymocytes from Nck.T⁺/⁺ (N = 6; top) and Nck.T⁻/⁻ mice (N = 6; bottom) were stained with the CD3ε.PRS-specific APA1/1 mAb, under resting conditions (0 min; shaded histogram) and 10 (solid line) or 30 (broken line) minutes after CD3ε engagement (10μg/ml; right). Isotype controls under resting (dotted line) and stimulating conditions (not shown) had superimposable profiles.

**Suppl. Fig. 12: Nck deletion results in increased CD3ε phosphorylation under resting and activated conditions.** Equal numbers of freshly isolated thymocytes (30 x 10⁶) from Nck.T⁻/⁻ and Nck.T⁺/⁺ mice were left untreated or underwent short stimulation (2min), by cross-linking of biotinylated αCD3ε/αCD8α Abs. Immunoprecipitation was performed on equal protein amounts with CD3ε– or pTyr-specific Abs. Immune complexes were resolved on a 15% SDS-PAGE gel. Immunoblotting was performed with biotinylated Abs, revealed by streptavidin-HRP. IgL results from the biotinylated Abs used for stimulation. Numbers represent the –fold increase of the relative signal intensity over wild type resting thymocytes.
Suppl. Fig. 13: *Nck is crucial for optimal ERK phosphorylation in the thymus.* Thymocytes freshly isolated from Nck.T⁺/⁺ and Nck.T⁻/⁻ mice were stimulated upon cross-linking of biotinylated αCD3ε/αCD8α (10μg/ml). Whole cell lysates were resolved by SDS-PAGE and immune-blotted with anti-pLAT (Tyr191, polyclonal Abs, Millipore) or pERK (Thr202/Tyr204, E10, Cell Signalling). Membranes were stripped and reprobed for β-actin (AC-15, Sigma) or total ERK1/2 (Rabbit polyclonal anti-serum, Cell Signalling) as loading controls, respectively. Phosphorylation of LAT (A), ERK1 (B) and ERK2 (C) is depicted as relative -fold increase of signal intensity over loading control. Data are representative of three independent experiments.
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**Image:**
- Western blot of Thymus with IB: Nck and IB: a-tubulin.
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B) Stimulus: αCD3ε/αCD8α

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C) Stimulus: αCD3ε/αCD8α

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